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Bacillus subtilis RarA acts as a positive RecA accessory protein

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Abstract

Ubiquitous RarA AAA^+ ATPases play crucial roles in the cellular response to blocked replication forks in pro- and eukaryotes. Here, we provide evidence that RarA regulates the activity of the central player in homologous recombination (HR), RecA, in response to DNA damage. During unperturbed growth, absence of RarA reduced the viability of Δ*recA,* Δ*recO* and *recF*15 cells, and during repair of H_2O_2- or MMS-induced DNA damage, *rarA* was epistatic to *recA*, *recO* and *recF*. Conversely, the inactivation of *rarA* partially suppressed the HR defect of mutants lacking end-resection (Δ*addAB*, Δ*recJ*, Δ*recQ*, Δ*recS*) or branch migration (Δ*ruvAB*, Δ*recG*, Δ*radA*) activity. RarA contributes to RecA thread formation, that are thought to be the active forms of RecA during homology search. The absence of RarA reduced RecA accumulation, and the formation of visible RecA threads *in vivo* upon DNA damage. When Δ*rarA* was combined with mutations in genuine RecA accessory genes, RecA accumulation was further reduced in Δ*rarA* Δ*recU* and Δ*rarA* Δ*recX* double mutant cells, and was blocked in Δ*rarA recF*15 cells. These results suggest that RarA contributes to the assembly of RecA nucleoprotein filaments onto single-stranded DNA (ssDNA), in concert with RecF, and possibly antagonizes RecA filament disassembly by RecX or RecU.

Introduction

During DNA replication, the forks encounter obstacles that can block their progression, and replication impairment is recognized as an important source of genetic instability (1-3). Maintenance of genome stability is one of the crucial functions in life. As a consequence, numerous and diverse mechanisms have evolved to minimize the frequency or impact of replicative stress (1, 2, 4, 5). Eukaryotic Mgs1/WRNIP1 and prokaryotic RarA, which are evolutionarily conserved AAA^+ ATPases associated with a variety of cellular activities, play important but poorly understood roles in cellular responses to stalled or collapsed replication forks (6-17).

Previous assays have indicated a poorly understood role for bacterial RarA in homologous recombination (HR). Inactivation of *Bacillus subtilis rarA* renders cells very sensitive to H_2O_2, but not to methyl methane sulfonate (MMS) or the UV radiation-mimetic compound 4-nitroquinoline-1-oxide (17). Similarly, an *Escherichia coli* null *rarA* (Δ*rarA*) mutant strain remains as capable of repairing UV-induced DNA damage as wild-type (*wt* or rec^+) cells (6, 9). In both bacteria *E. coli* and *B. subtilis* the viability under unperturbed conditions of *E. coli* and *B. subtilis* Δ*rarA* Δ*recA* cells is significantly lower than that of the Δ*recA* control (9, 17). Since the *recA* gene is not epistatic with functions involved in base or nucleotide excision repair, but the *E. coli* or *B. subtilis rarA* gene is epistatic to *recA* in response to DNA damage (9, 17), we assume that RarA is a genuine repair-by-recombination protein. Unless otherwise stated, the indicated genes and products are of *B. subtilis* origin.

Bacterial RarA shares sequence homology with DnaX, a subunit of the clamp loader complex, and with RuvB, a subunit of the RuvAB branch migration translocase (6), but *B. subtilis* RarA could not substitute for DnaX in the cognate reconstituted *in vitro* DNA replication system (15). Rather, these assays showed that RarA inhibited initiation of PriA-dependent DNA replication, but not chain

elongation, suggesting that RarA might impede the assembly of the replicative helicase and prevent that recombination intermediates contribute to pathological DNA replication restart (15). RarA exerts its action through its interaction with the essential SsbA (counterpart of *E. coli* SSB [SSB_{Eco}]) and with PriA proteins (15). In addition to RarA, SsbA protein interacts with various recombination (RecQ, RecS, RecJ, RecG, RecO, RecD2, SbcC and SbcE) and replication (PriA, DnaG and DnaE) proteins, of which RecS, RecD2, SbcE and DnaE are absent in *E. coli* cells (18). These data suggest a role of RarA in recombination-dependent DNA replication, although RarA might follow different avenues in distantly related bacteria (14-17). For example, when DNA replication is blocked, upon dNTPs depletion by hydroxyurea, $RarA_{Eco}$ foci disassemble from the replication fork and disappear (19). However, *in vitro* studies suggested that $RarA_{Eco}$ may contribute to replication fork rescue by creating a flap on the lagging strand, so that the replicative helicase and its associated replisome could continue chain elongation without the need for replisome disassembly and replication restart (14). In *B. subtilis* cells, inhibition of the replicative DNA polymerase PolC, by the specific inhibitor *p*-hydroxyphenylazo-uracil (HPUra), confines the RarA molecules towards the collapsed replication forks (17). In this bacterium it was shown that *B. subtilis* RarA-mVenus transiently colocalizes with the DnaX-CFP protein, and it alternates between static and dynamic states. RarA is confined to the replication forks when the preprimosomal DnaB protein is non-functional, but the opposite occurs upon inactivation of the replicative DNA helicase (DnaC) (16, 17), revealing an intricate function related to DNA replication restart.

RarA forms mobile foci, usually one per cell containing many molecules, that move in a time scale of minutes in ~50% of total cells, mostly close to replication forks, in which RarA is likely DNA-bound. On a time scale of milliseconds, ~50% of RarA molecules move very slowly or are static, likely within the slowly moving foci, while the remaining fraction was highly dynamic, diffusing throughout the cells (16, 20). DNA damages changed the ratio of static (DNA-bound) and

freely diffusive RarA, *e.g*. H_2O_2 decreased the static subpopulation of RarA at the replication forks, and instead, RarA was recruited to areas located away from the replication forks. Exposure to H_2O_2 increased the fraction of dynamic molecules, but not treatment with MMS, and this was exacerbated by the absence of end resection or Holliday junction (HJ) processing proteins (16). The number of cells containing slowly moving RarA foci was also affected by several proteins acting in homologous recombination (HR) (16), indicating that the number of molecules acting within the foci, and the positioning of the foci, is affected by interactions with HR proteins.

To analyze the role of RarA in repair-by-recombination at the genetic level, the Δ*rarA* deletion was moved into *rec*-deficient strains impaired in DNA end resection (*addAB*, *recQ*, *recS*, *recJ*), RecA mediators (*recO*) and/or modulators (*recF*, *recX*, *recU*), or HJ processing and cleavage/dissolution (*recG*, *ruvAB*, *radA*, *recU*, *recQ*, *recS*). Also, the relation to the DNA repair defect of the poorly characterized *recD*2 mutation (21) was investigated. In this study, we show that lack of RarA reduced cell viability in the Δ*recO* and Δ*recA* and in less extent of the *recF15* context, but these mutant strains were equally sensitive to H_2O_2- or MMS-induced non-bulky DNA lesions of oxidative nature (epistasis). The absence of RarA partially suppressed the DNA repair defect of cells impaired in DNA end resection (*addAB*, *recQ*, *recS*, *recJ*), or HJ processing and cleavage/dissolution (*recG*, *ruvAB*, *radA*, *recU*, *recQ*, *recS*), as well as the DNA repair defect of the *recD*2 mutation. Lack of RarA might reduce the accumulation of the signal (RecA filament formation) that is considered to facilitate LexA self-cleavage as judged by the drop of RecA levels upon exposure to increasing mitomycin C (MMC) concentrations and the reduced number of RecA threads in Δ*rarA* cells. Together, these data suggest that RarA controls RecA filament growth and might counteract negative mediators RecX and/or RecU.

Materials and Methods

Bacterial strains

B. subtilis BG214 and its isogenic derivatives are listed in Table 1. The null *rarA* (Δ*rarA*) mutation was transferred into the other genetic backgrounds by SPP1-mediated chromosomal transduction. The *recF*15 point mutation and a null mutation in *recF* (Δ*recF*) are equally deficient in DNA repair, but the latter showed a reduced cell fitness, because it compromises expression of the downstream essential *gyrB* and *gyrA* genes, thus we worked with the inactive *recF*15 strain (22). In RecF15 the highly conserved negatively charged residue E255 is replaced by a positively charged one K255, RecF E255K (22). The accuracy of the double mutations was analyzed by PCR amplification and nucleotide sequence analyses.

Survival studies

H_2O_2, MMS and MMC were obtained from Sigma Aldrich (Germany). The sensitivity of cells to acute exposure to MMS or H_2O_2 was determined by growing *rec*$^+$ and its isogenic derivative strains (see Table 1) in NB to an OD_{560} = 0.4 at 37 ºC with agitation. Then, cells were incubated with increasing concentrations of MMS or H_2O_2 for *15 min. Treated cells were diluted and plated on NB agar plates,* incubated overnight (ON) at 37 ºC, *and the* colonies forming units/ml (CFUs/ml) were counted. The large majority of cells were one and two non-separated with an average of ~1.6 cells/CFU, thus we have assumed an acceptable correlation of OD_{560} with CFUs.

Cell staining

The LIVE/DEAD BacLight bacterial viability kit was purchased from Fisher Scientific was used. Cells were exponentially grown in NB to an OD_{560} = 0.4 at 37 ºC with agitation for *30 min*. Appropriate dilutions were stained with membrane-permeant SYTO 9, which labels living bacteria with green fluorescence, and then with membrane-impermeant propidium iodide (PI), which stains

127 cells with a membrane compromised defect with red fluorescence, and subjected to conventional
128 direct count of total cells using a fluorescence microscope and appropriate filters (470 ± 20 nm
129 excitation filter and 515 ± 20 nm emission filter for both SYTO 9 and PI), as reported (21). When
130 cells are permeant to PI, its counterstaining activity competes with SYTO 9 for binding to DNA,
131 and SYTO 9 staining signal is not detected. In each experiment >1000 CFUs were counted.

132 ***RecA protein quantification***

133 For quantification of RecA induction of the *recA* gene expressed from its native locus and promoter,
134 cells were grown in NB to an OD_{560} = 0.4 at 37 ºC with agitation and treated with increasing MMC
135 concentrations (0.07 to 1.5 μM) for 30 min. Cells (2 ml) were centrifuged, resuspended in 100 μl of
136 buffer A (50 mM Tris HCl, pH 7.5, 1 mM DTT, 5% glycerol) containing 300 mM NaCl and lysed
137 by sonication. Extracts from each experimental condition, containing similar concentrations of total
138 and housekeeping proteins, were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide
139 gel electrophoresis (PAGE) alongside the purified RecA protein standard (10 to 500 ng) as reported
140 (23). Gels were transferred, and Western blots were developed with rabbit polyclonal anti-RecA
141 antibodies (23). This antibody showed no signal in the absence of RecA, suggesting that no cross-
142 reactive signal interferred in our studies.

143 RecA protein bands on developed immunoblots were quantified with a scanning densitometer
144 (ImageLab software, BioRad). Purified RecA protein standard yielded a linear relationship between
145 antibody signal and the RecA protein concentration. The amount of RecA protein in each induced
146 sample was interpolated from the standard curve performed with known amounts of purified protein,
147 as described previously (23). The *in vivo* concentration of RecA was estimated considering the cell
148 volume of 1.2 femtoliters, and the amounts of cells loaded in the gel, based on the total number of
149 CFUs.

150

151 **Fluorescence microscopy and data analysis**

152 A C-terminal fusion of the fluorescent protein mVenus to RecA was generated by cloning the 3′-
153 end 500-bp of *recA* (excluding the stop codon) into plasmid pSG1164 mVenus (24), which was
154 integrated into the *recA* gene locus on the *B. subtilis* chromosome by single crossover
155 recombination. Epifluorescence microscopy was used to monitor filament formation and dynamics
156 of RecA before and after stress conditions at 30 °C (OD_{600} = ~ 0.3). Cells were treated with 0.5 mM
157 H_2O_2 (obtained from Sigma Aldrich) or were not treated. For fluorescence microscopy, *B. subtilis*
158 cells were grown in $S7_{50}$ minimal medium at 30 °C under shaking conditions until exponential
159 growth, using a Zeiss Observer Z1 (Carl Zeiss) with an oil immersion objective (100× magnification,
160 NA 1.45 alpha Plan-FLUAR) and a CCD camera (CoolSNAP EZ, Photometrics). Electronic data
161 were processed using Metamorph 7.5.5.0 software (Molecular Devices, Sunnyvale, CA, USA),
162 which also allows the calibration of the fluorescence intensity and pixel size to determine the cell
163 length and BacStalk (25), time-lapse epifluorescence microscopy of RecA-mV were collected every
164 5 min.
165

Results and Discussion

Δ*rarA* reduces viability in Δ*recO* and Δ*recA*

To better understand the role of RarA in repair-by-recombination, a *rarA* deletion (Δ*rarA*) was combined with *rec*-mutations in DNA end resection (*addAB*, *recQ*, *recS*, *recJ*), RecA mediators (*recO*) and/or modulators (*recF*, *recX*, *recU*), and HJ processing and cleavage/dissolution (*recG*, *ruvAB*, *radA*, *recU*, *recQ*, *recS*), as well as the DNA repair defect of the poorly characterized *recD*2 mutation (Table 1). The *recA*, *recF*, *recO*, *recG*, *recJ*, *recQ, recR*, *ruvA*, *ruvB*, *radA* and *rarA* genes have their counterpart in *E. coli* genes with identical name, the *addAB* and *recU* genes have their counterpart in the $recBCD_{Eco}$ and $ruvC_{Eco}$ and the *recS* and *recD*2 genes are absent in *E. coli* (26, 27). In contrast, *B. subtilis* cells lack the RecA modulators *dinI* and *rdgC* (28).

If a DNA damage is not removed, DNA replication is not able to continue to completion and the cell will not survive. It has recently been reported that *B. subtilis* replisome dissociation occurs at a frequency of ~5-fold events per replisome, per cell cycle (29). Interestingly, even in the absence of any external DNA damage the viability of the constructed strains listed in Table 1 was quite different. The combination of Δ*rarA* with Δ*addAB*, Δ*recS*, Δ*recQ* or Δ*recJ* (impaired in alternative pathways of end-processing), Δ*recX* (negative modulator), Δ*radA* (impaired in branch migration translocase) or with Δ*recD2* (yet unclassified) (23, 30-32) yielded similar or only slightly reduced (<1.4-fold) viability relative to rec^+ cells (Figure 1A). Thus, additional deletion of RarA in these mutant backgrounds having similar viability as *wt* cells (21, 32, 33) appeared to be associated with a low fitness cost.

At mid-exponential phase the number of CFUs was reduced 5- to 7-fold in Δ*recU*, Δ*ruvAB* or Δ*recG* (impaired in translocation of branched structures and HJ resolutution) cells, compared to the rec^+ control (21, 33), and the viability of the double (Δ*recU* Δ*rarA*, Δ*recG* Δ*rarA*) or triple (Δ*ruvAB* Δ*rarA*) mutant strains was marginally affected, as shown in Fig. 1A. These findings suggest that the

deletion of *rarA* does not cause an extra fitness cost when compared to the Δ*recU*, Δ*recG* or Δ*ruvAB* cells. The viability of the Δ*radA* cells was similar to or slightly reduced (<1.5-fold) relative to *rec*$^+$ cells, but the viability of the Δ*rarA* Δ*radA* double mutant strain was reduced ~10-fold compared to the *rec*$^+$ strain (Fig. 1A), suggesting that absence of the RarA and so far less well characterized RadA/Sms functions poses a considerable threat for cell viability.

Previous studies demonstrated that in the absence of any external DNA damage, the Δ*recO* and *recF*15 mutations (impaired in RecA nucleation and filament formation) only slightly affect (<1.4-fold) the number of CFUs at mid-exponential phase (Fig. 1B), but the Δ*recA* mutation lead to a strong reduction (~10-fold) (17, 34). Interestingly, the absence of RarA caused a ~15-, ~60- and ~145-fold reduction in the number of CFUs at mid-exponential phase in the Δ*rarA recF*15, Δ*rarA* Δ*recO* or Δ*rarA* Δ*recA* backgrounds, respectively, compared to the Δ*rarA* single mutant strain (Fig. 1B). Thus, there is a strong synthetic defect of combining the Δ*rarA* deletion with loss-of function in RecA accessory proteins or most severely with loss of RecA itself. Similarly, the *E. coli* Δ*rarA* Δ*recA* cells have low viability when compared to Δ*recA* cells (9), revealing a strong parallel in this aspect.

Moving on with our analyses, we chose the double mutant strains with the lowest viability (Δ*recO* Δ*rarA* and Δ*recA* Δ*rarA*). To investigate whether this reduced viability in Δ*recO* Δ*rarA* and Δ*recA* Δ*rarA* correlates with membrane-compromised cells, two different fluorophores were used (SYTO 9 and PI). Exponentially grown cells (OD_{560} = 0.4) were stained with SYTO 9 (in green) and PI (in red). The proportion of exponentially growing *rec*$^+$ and Δ*rarA* cells stained with PI (membrane compromised/dead) was low (~1% and ~1.8% of total cells, respectively). The proportion of Δ*recO* and Δ*recA* cells stained with PI was 9.8% and 5.6% of total cells, respectively (Fig. 1C). The absence of RarA increased the proportion of PI stained cells by only ~1.2 fold in Δ*recA* cells, but the number increased by ~4-fold in the Δ*recO* background (Fig. 1C). Thus, the

strong decrease in CFUs in Δ*recA* Δ*rarA* cells (~145-fold) does not correlate with the number of membrane compromised cells (7.1% of total cells), but it partially does in Δ*recO* Δ*rarA* cells (~60-fold reduction in CFUs versus 39.2% PI staining cells) (Fig. 1C). These results show that Δ*recO* Δ*rarA* and Δ*recA* Δ*rarA* double mutant strains show a gross cell proliferation defect (Fig. 1B) and that RecO is crucial to alleviate the membrane compromise defect (Fig. 1C).

Experimental approach for repair-by-recombination studies

To gain further insight into the involvement of RarA in repair-by-recombination the double (triple in case of Δ*rarA* Δ*addAB* or Δ*rarA* Δ*ruvAB*) mutant strains were exposed to DNA damaging agents, for 15 min, at concentrations that are bacteriostatic to *rec*$^+$ cells growing in nutrient broth (NB) medium. MMS and H_2O_2 were chosen, because both induce modifications in DNA bases, but in the presence of Fe(II), H_2O_2 treatment additionally generates DNA nicks (35). MMS- or H_2O_2-damaged bases are mainly repaired by direct DNA damage reversal, such as the guanine oxidation prevention/repair system, base excision repair or mismatch repair (35-37). Unrepaired MMS- or H_2O_2-lesions primarily halt elongation by the replicative DNA polymerase, and thereby stall replication fork progression. Stalled forks can be repaired by different repair-by-recombination or postreplication repair pathways (36-38). The H_2O_2 generated nicks collapsed replication forks, and these intermediates can be repaired by different repair-by-recombination pathways (1, 27, 39). Our previous work showed that RarA single mutants are very sensitive to H_2O_2-, but resistant to MMS-induced lesions (17), showing that RarA deals differently with the effect of the two drugs.

We classified the different outcomes into "moderately sensitive" when the viability was reduced less than 10^2-fold, into "sensitive" when it was reduced less than 10^3-fold, into "very sensitive" when viability was reduced from more than 10^3-fold and up to 10^5-fold, and when the viability was

reduced more than 10^5-fold the mutant strain was considered "extremely sensitive" to the damaging agent.

RarA is not required for end-resection but affects the outcome of repair events in end-resection mutants

In *B. subtilis,* there are two alternative DNA end resection pathways: the AddAB complex, and RecJ single-stranded exonuclease in concert with a RecQ-like DNA helicase (RecQ or RecS) (40). Both machineries contribute to the processing of 5′-termini at both ends of the break, generating a 3′-tailed duplex intermediate that is the substrate for RecA nucleation and filament growth, and the latter also resects single strand gaps (27, 41). The lack of AddAB and RecJ renders cells extremely sensitive to DNA-damaging agents, with a sensitivity similar to that of Δ*recA* cells (40), showing that HR is no longer operative in their absence. In our experiments, Δ*addAB* mutations rendered cells very sensitive and the Δ*recS*, Δ*recQ* and Δ*recJ* mutations cells sensitive to H_2O_2 or MMS exposure (Fig. 2A and 3A) (40), suggesting a certain hierarchical order in the processing of the broken molecules by the AddAB or RecJ-RecQ(RecS) complexes.

The acute lethal H_2O_2 dose that reduced Δ*rarA* cells survival by 99% (LD_{99}) was ~0.38 mM (Table 2). A Δ*rarA* mutation rendered cells very sensitive to acute exposure to H_2O_2, with an LD_{99} >16-fold lower than for the *rec*$^+$ control (Fig. 2, Table 2) (17). Curiously, the survival rate of Δ*addAB* Δ*rarA* cells was increased ~12-fold when compared to the parental Δ*rarA* or Δ*addAB* strains (Fig. 2A, Table 2), suggesting that in the absence of both RarA and AddAB the recombinational intermediates are channelled towards another repair pathway(s). The DNA repair defect of *rarA* mutant cells was also partially suppressed when the mutation was combined with *recQ* or *recS*, resulting in an LD_{99} to H_2O_2 that was ~5-fold higher than that of Δ*rarA* cells (Fig. 2A, Table 2).

258 Thus, Δ*addAB*, Δ*recQ* or Δ*recS* mutations suppressed the DNA repair defect of the Δ*rarA* mutation
259 upon exposure to H_2O_2.

260 The connection between *rarA* and *recJ* mutations was somewhat different than expected with
261 regard to the above mentioned mutations. The survival rate of Δ*recJ* Δ*rarA* was reduced ~9-fold
262 compared to Δ*recJ*, and the LD_{99} was comparable to that of the Δ*rarA* control (Fig. 2A, Table 2).
263 At a higher H_2O_2 dose a different outcome was observed. At 2 mM H_2O_2 the survival rate increased
264 ~4-fold, and at 4 mM of H_2O_2 the survival of the Δ*recJ* Δ*rarA* mutant strain increased ~17-fold
265 compared to the Δ*rarA* control (Fig. 2A), suggesting that the absence of *recJ* partially suppressed
266 the DNA repair defect of Δ*rarA* cells. The differences observed between the *recJ* and the other
267 functions involved in end-pprocessing in combination with Δ*rarA* could be due to the different
268 activities. RecJ is involved in base excision repair, methyl-directed mismatch repair and repair-by-
269 recombination (27, 39, 42), whereas no role other than repair-by-recombination has been described
270 for AddAB, RecQ or RecS (26, 27). In none of the cases of double mutant cells, we observed an
271 epistatic effect, nor strong synergistic effects. Therefore, we have to assume that RarA is not
272 required for end resection.

273 To further evaluate the contribution of RarA to end resection, exponentially growing cells were
274 acutely exposed to increasing MMS concentrations for 15 min (Fig. 3). The acute LD_{99} dose for
275 MMS for *rec*$^+$ cells (10 mM) was lower than that for Δ*rarA* cell (>50 mM) (Table 2), confirming
276 that in the absence of RarA, cells remain recombination proficient, and apparently more capable of
277 repairing MMS-induced DNA damage than *wt* cells (17). AddAB cells were very sensitive to MMS,
278 but the additonal mutation in *rarA* rescued this phenotype: the LD_{99} to MMS was increased by ~55-
279 fold in Δ*addAB* Δ*rarA* cells relative to the Δ*addAB* mutant strain (Fig. 3A, Table 2). The survival
280 rate in Δ*recS* Δ*rarA*, Δ*recJ* Δ*rarA* or Δ*recQ* Δ*rarA* was enhanced ~2-fold when compared to the
281 single Δ*recS*, Δ*recJ* or Δ*recQ* strains (Fig. 3A, Table 2). These findings suggest that inactivation of

rarA makes canonical DSB repair deleterious for cell survival, because the absence of functions involved in long-range 5′→3′ end resection (e.g., AddAB, RecJ, RecQ, RecS) partially suppressed the DNA repair defect of Δ*rarA* cells in response to H_2O_2 or MMS (Fig. 2A and 3A). This is in agreement with a previous report showing that WRNIP1 is directly involved in preventing uncontrolled MRE11-mediated degradation of stalled replication forks (13). The observed genetic interactions are in line with the observation that exponentially growing Δ*addAB*, Δ*recS*, Δ*recQ* or Δ*recJ* cells show strongly reduced RarA mobility (16), *i.e*. the activity of RarA with respect to its binding to DNA is considerably altered in end-resection mutants.

Branch migration or HJ processing of recombination intermediates activities do not require RarA, but their loss partially suppress *rarA* phenotypes

A branch migration translocase binds to HJs, formed as HR intermediates (double-HJ), or when replication forks stall and reverse (HJ-like structure), and promotes HJ migration (43-45). When its cognate site becomes available, the RecU resolvase cleaves the double-HJ, in concert with the RuvAB translocase, to preferentially generate non-crossover products, and rarely crossover products (postsynaptic step) (27, 42, 44-46). It is unknown whether RecU can cleave the reversed forks generated by RecG in *B. subtilis*. In any event, RecU has two activities: to mediate HJ cleavage in concert with a branch migration translocase (47), and to modulate RecA nucleoprotein filament formation (48, 49).

In our assays, the Δ*recG*, Δ*ruvAB* and Δ*recU* mutations rendered cells very sensitive and the Δ*radA* mutation sensitive to H_2O_2 or MMS exposure (Fig. 2B and 3B) (21, 33, 50, 51). The survival rate to H_2O_2 of Δ*radA* Δ*rarA* or Δ*ruvAB* Δ*rarA* mutant cells was increased compared to the less sensitive single mutant strain, with an LD_{99} to H_2O_2 ~12-fold or ~3-fold higher than the Δ*rarA* strain, respectively (Fig. 2B, Table 2). The LD_{99} to H_2O_2 of the Δ*recG* Δ*rarA* or Δ*recU* Δ*rarA* mutant

strains was similar to the more sensitive single mutant strain (Fig. 2B, Table 2). However, at a H_2O_2 dose as high as 2 mM, the survival rate of Δ*recG* Δ*rarA* or Δ*recU* Δ*rarA* mutant strains increased ~16-fold and ~25-fold relative to the Δ*rarA* strain (Fig. 2A), suggesting that Δ*recG* or Δ*recU* partially suppressed the DNA repair defect at high H_2O_2 concentrations. When cells were acutely exposed to increasing MMS concentrations (Fig. 3B), the sensitivity of Δ*recU* Δ*rarA*, Δ*recG* Δ*rarA* and Δ*radA* Δ*rarA* cells to MMS was lower than that of the single mutants, with LD_{99} to MMS of ~2-, ~2- and ~12-fold higher than the Δ*radA*, Δ*recG* and Δ*recU* mutant strains, but the LD_{99} of the Δ*ruvAB* Δ*rarA* cells was similar to that of the Δ*ruvAB* strains (Fig. 3B). At MMS doses as high as 20 mM, the survival rate of Δ*ruvAB* Δ*rarA* mutant strain increased ~3-fold compared to the Δ*ruvAB* control (Fig. 2A), suggesting that Δ*rarA* partially suppressed the DNA repair defect of Δ*ruvAB* cells at moderate MMS concentrations.

Taken together, it can be stated that i) the absence of RuvAB, RecG, RadA/Sms or RecU partially suppressed the acute sensitivity to high H_2O_2 concentrations of Δ*rarA* cells (Fig. 2B); ii) the absence of RarA partially suppressed the repair defect seen in the absence of the branch migration translocase (RadA/Sms) or of the HJ resolvase (RecU) upon exposure to MMS, but not of RuvAB or RecG (Fig. 3B). This is consistent with the observation that in the absence of HJ-processing enzymes, the static RarA population decreases in *ruvAB*, *recG* and *radA* cells, meaning that RarA is less often bound to DNA, but increased in *recU* cells (16), i.e. RarA becomes more engaged with DNA in cells lacking RecU.

RarA is epistatic to RecO and RecF in response to DNA damage

In vitro, *B. subtilis* RecA cannot nucleate on the SsbA-ssDNA complexes, and AddAB cannot activate RecA to catalyze DNA strand exchange (52). The two-component mediator SsbA and RecO (in conjunction with RecR), together with positive (RecF) and negative modulators (RecX, RecU),

load RecA on a ssDNA gap or a 3′-tailed duplex ssDNA, facilitate RecA filament growth and activate RecA to catalyze DNA strand exchange *in vitro*, with SsbA, RecO, RecR, RecF and RecX collectively acting *in vivo* (23, 53-55).

As previously shown, *recF*15 and Δ*recO* mutations rendered cells very sensitive to H_2O_2 or MMS exposure (Fig. 2C and 3C) (22, 56). The double Δ*recO* Δ*rarA* or *recF15* Δ*rarA* mutant strains were equally sensitive to H_2O_2 or to MMS as the more sensitive single mutant strain, suggesting epistasis (Fig. 2C, 3C and Table 2). This is consistent with the observation that *rarA* is epistatic to *recA* in response to H_2O_2- or MMS-induced DNA damage (17). Moreover, the ratio of DNA bound to freely moving RarA is altered in Δ*recO* or *recF*15 cells upon exposure to DNA damaging agents (16), showing that the genetic interaction is reflected in the presumed activity of RarA. As described for *B. subtilis rarA* (Fig. 2C, 3C), eukaryotic WRNIP1 functions in the same pathway as the Rad51 mediator BRCA2 (13).

Δ*rarA* partially suppresses the DNA repair defect of Δ*recD2* or Δ*recX* cells to treament with H_2O_2

The negative modulator RecX has been shown to disassemble RecA nucleoprotein filaments (23, 55), and preliminary data from our laboratory has suggested a similar role for RecD2, whose funstion in HR is poorly understood (32, 57). Investigating the genetic connection between RarA and RecX or RecD2, we found Δ*recX* and Δ*recD2* mutants to be sensitive to acute H_2O_2 or MMS exposure (Fig. 2D and 3D), as described earlier (23, 32). The LD_{99} to H_2O_2 of the Δ*recD2* Δ*rarA* or Δ*recX* Δ*rarA* double mutant strain was not significantly different that the Δ*rarA* strain (Fig. 2D, Table 2). However, at a H_2O_2 dose as high as 2 mM, the survival rate of Δ*recX* Δ*rarA* or Δ*recD2* Δ*rarA* mutant strain was increased ~4-fold or ~100-fold, respectively, compared to the Δ*rarA* control (Fig. 2A), suggesting that Δ*recX* and Δ*recD2* partially suppress the DNA repair defect in the Δ*rarA* context at

high H_2O_2 concentrations. With respect to MMS treatment, the Δ*recD*2 mutation partially suppressed the DNA repair defect of Δ*recD*2 Δ*rarA* cells (Fig. 3D, Table 2), whereas the Δ*recX* Δ*rarA* strain was slightly more sensitive to MMS than the single Δ*recX* mutant strain (Fig. 3D, Table 2). Thus, while the *rarA* deletion has a suppressor phenotype to high H_2O_2 concentrations with regards to *recX* and *recD2* deletions, Δ*recX* Δ*rarA* cells show higher sensitity to MMS treatment than the Δ*recX* control (Fig. 3D). Interestingly, RarA dynamics decreased in the Δ*recX* strain (RarA was more stronlgy bound to DNA than in *wt* cells), and the opposite behaviour was observed in the Δ*recO* or *recF*15 backgrounds (16). Thus, there is a strong connection between RecX and RarA in a genetic and cell biological aspect.

The threshold for maximal RecA levels after DNA damage is increased in Δ*rarA* cells

The previous results suggest that RarA has two roles: it may protect DNA from deleterious action of recombination proteins, and additionally it may work as a RecA accesory protein. *In vitro, B. subtilis* RecA·ATP cannot nucleate onto SsbA coated ssDNA, and cannot catalyze DNA strand exchange between circular ssDNA and linear duplex in the absence of accessory factors (52, 58, 59). Thus, RecA activity is regulated by accessory proteins (28). Accessory factors can be divided into two general groups: mediators that act before and the modulators that act during homology search and the DNA strand exchange reaction (presynaptic step) (27, 28). Mediators and modulators can be further divided into two classes, acting positively or negatively on RecA nucleation and/or filament growth (60). The mediators and modulators are partially conserved between *B. subtilis* and the genetically distant *E. coli*. For example, DinI, which antagonizes the role of RecX, and RdgC, which inhibits RecA-dependent LexA autocleavage, are missing in *B. subtilis* cells. Also different from *E. coli*, none of the *B. subtilis* mediators and modulators are part of the SOS response (27, 61).

373 Damages in the DNA template block DNA replication in a concentration dependent manner,
374 leading to extended ssDNA regions coated by SsbA. *B. subtilis* RecA·ATP acts as a sensor of
375 excessive ssDNA, and with the help of mediators, it assembles onto the SsbA-coated ssDNA to
376 generate RecA* (a right-handed RecA·ATP nucleoprotein filament) that conducts all the catalytic
377 steps of HR (23, 53, 54)), with the help of the RecF, RecX and RecU modulators (23, 53, 54). Then,
378 different dynamic RecA* filaments chaperone the LexA transcriptional repressor, and facilitate its
379 auto-cleavage (62), thereby de-repressing ~33 genes (*recA* among them) (61), and activating the
380 SOS response (63). A more general RecA-dependent DNA damage response is triggered following
381 MMC-induced replication arrest, with ~140 genes showing altered expression, including LexA-
382 dependent (*e.g.*, *ruvA* gene) and LexA-independent (*e.g.*, *recN* gene) genes (64, 65).
383 Exponentially growing cells were estimated to contain ~4,800 RecA monomers/CFU as judged
384 by Western blot (Fig. 4A) and by integrated mass spectrometry and 2-D gel-based proteomics
385 analyses (66). This is good agreement with the literature (23, 65). In rec^+ cells, RecA reached its
386 maximal level of expression at ~0.6 μM MMC, and its maximal induction caused a ~5-fold increase
387 to 26,000 ± 1,000 RecA/CFU (Fig. 4A), similar to what was shown before (26,000 ± 1,000) in the
388 wt as well as in the Δ*lexA* background (23, 65), suggesting that this MMC concentration provides
389 the DNA damage threshold necessary to fully de-repress RecA expression. Under similar
390 experimental conditions, *recA* promoter utilization increased 6- to 10-fold (67). For comparison,
391 undamaged *E. coli* cells have 7,000 - 15,000 RecA monomers/cell and these levels increase to
392 ~100,000 RecA/cell upon DNA damage (68). When MMC was replaced H_2O_2, similar RecA
393 expression levels were observed, but here the correlation between RecA accumulation and H_2O_2
394 concentrations were less pronounced (65).
395 Two different outcomes can be envisioned upon addition of increasing MMC concentrations in
396 the absence of a RecA mediator or modulator. First, in the absence of a mediator or a positive

397 modulator, a negative RecA modulator will promote a net RecA filament disassembly, with
398 subsequent reduction in the probability of LexA repressor autocleavage. Thus, a higher MMC dose
399 should be required to reach maximal RecA expression levels. Secondly, in the absence of negative
400 modulators, the positive mediators and/or modulators will facilitate RecA filament assembly, so that
401 the probabilities of RecA filament increase as well as the interaction with LexA. Thus, a lower dose
402 of DNA damage should be sufficient for RecA to stimulate LexA auto-cleavage, so maximal RecA
403 levels are obtained at lower MMC doses in the absence of negative regulators. For example, in the
404 absence of the positive modulator RecF, an MMC dose higher than the one needed in the *rec*$^{+}$ control
405 was required to maximal RecA expression levels, but in the absence of negative modulator RecX, a
406 lower MMC dose was sufficient (Fig. 4A) (23, 55).

407 We then tested whether RarA contributes to RecA nucleoprotein filament formation and
408 compared its RecA levels with that in the absence of RecO (positive mediator) or RecF (positive
409 modulator). In uninduced Δ*rarA*, Δ*recF*15 or Δ*recO* cells, RecA levels were maintained at a similar
410 basal level estimated to be 4,600 ± 1,200 RecA monomers/CFU during mid-log phase of cell growth
411 (Fig. 4A). The absence of RarA reduced maximal RecA levelsn (from ~26,000 to 16,000 ± 900
412 RecA/CFU) that were reached at ~0.75 μM MMC, and did not barely change at 1.5 μM MMC (Fig.
413 4A). Similarly, a higher MMC dose is necessary to facilitate maximal RecA expression in cells
414 impaired in the RecF modulator, but no increase is observed in cells lacking RecO (Fig. 4A) (23).
415 Because RarA and RecO both interacts with SsbA rather than with RecA (18), it is unlike that RarA
416 binds to the RecA filament and competes with LexA binding, preventing its autocleavage. Thus, we
417 can exclude this alternative explanation for a higher MMC dose required for maximal RecA
418 expression levels, suggesting that RarA is a true mediator or modulator of RecA, and that it faciliates
419 and/or stabilises RecA filaments onto ssDNA.

RarA is required for efficient RecA filament formation *in vivo*

To analyse whether RarA participates in RecA nucleation or facilitates RecA filament growth, we used a functional RecA-mVenus (mVenus is a variant of fluorescent protein YFP), for the visualization of RecA filaments (termed "threads") in live cells. The C-terminal fusion was integrated at the original gene locus, such that the fusion is the sole source of RecA expresed in cells, under the control of the original promoter. The RecA-mVenus fusion is repair proficient, as the RecA-mVenus strain was as viable as wild type cells after induction of DNA damage, in contrast to the highly sensitive *recA* deletion strain. RecA-mVenus changed from a localization pattern throughout the cells ("diffuse") or at discrete spots to form striking filemantous structures upon induction of DNA damage (Fig. 5). These filamantous structures have been described before (69) and were termed "threads", because it is still unclear if these structures correspond to RecA-ssDNA observed *in vitro*. Although evidence for this notion has been described (70), we will maintain the term "threads" to describe the structures observed by epifluorescence microscopy. Formation of RecA threads was maximal 40 min after induction of DNA damage, and thereafter, threads dissipated in favour of the diffuse or spot-lioke localization seen in the absence of DNA damage (Fig. 5). Strikingly, even at 40 min after addition of H_2O_2, Δ*rarA* mutant cells only showed the RecA patch- or spot-like structures that occasionally had short filamentous extensions (Fig. 6A). The failure to form discrete RecA threads can be most conveniently seen in the demographs (Fig. 6B), which do not reflect different levels of RecA-mVenus, but visualize the presence or absence of sharply contrasted fluorescent structures, *i.e.* RecA threads. In order to follow the dynamics of formation of RecA threads, we scored the number of cells containing diffusely localized RecA, RecA spots or RecA threads, during exponential growth (no damage) or in 10 min intervals following damage induction. Fig. 6C shows that while less than 10% of exponentially growing cells

443 contained visible RecA threads or spots (no damage), ~65% of cells contained RecA threads and
444 ~15% RecA spots as early as 20 min after addition of H_2O_2, which declined thereafter back towards
445 the pattern seen in untreated cells. In stark contrast, only a maximum of ~15% of Δ*rarA* cells
446 contained RecA threads, but ~60% RecA spots only. Assuming that the accumulation of RecA into
447 spots represents RecA loading events onto ssDNA, and the formation of threads extended filament
448 formation, we can propose that RarA plays an important role in the formation of RecA threads by
449 promoting the extension of filaments, stabilizing the RecA nucleprptein filament or by
450 downregulating the activity of negative modulators. Thus, RarA plays a dual role during HR, in
451 addition to its activity in replication re-initiation (15), it also strongly affects the formation of RecA
452 threads, which have been shown to be the active form of RecA during HR (69).

453 **RarA counteracts the action of RecU and RecX modulators**

454 Previously, it has been shown that inactivation of *recX* reversed the effect of the *recF*15 mutation
455 with regard to the level of RecA, with RecA levels comparable to *rec*$^+$ cells (23). We favour the
456 view that RarA acts as an antagonizer of RecX and/or of RecU. In the absence of MMC, RecA levels
457 were estimated to be 4,600 ± 1,200 RecA monomers/CFU in Δ*recU* cells (Fig. 4A). As expected for
458 a negative modulator, a significant net RecA accumulation was observed upon exposure to low
459 MMC concentrations in Δ*recU* cells. As low as 0.07 µM MMC already increased RecA levels, and
460 the maximal level of RecA accumulation was reached at ~0.3 µM MMC (26,000 ± 1,100
461 RecA/CFU) (Fig. 4A). Similar results were observed in the absence of the negative modulator RecX
462 (Fig. 4A) (23, 55). To test whether RarA may antagonize the action of RecX or RecU, the expression
463 levels of RecA were measured in Δ*recX* Δ*rarA* or Δ*recU* Δ*rarA* cells. The basal level of RecA in
464 the Δ*recU* Δ*rarA* and Δ*recX* Δ*rarA* strains was slightly lower than in the *rec*$^+$ cells (~4,100 RecA

monomers/CFU) (Fig. 4B). In the presence of increasing MMC, RecA expression in Δ*recU* Δ*rarA* or Δ*recX* Δ*rarA* cells was similar to *rec*$^+$ cells up to 0.15 μM MMC, but no further increase was observed at higher MMC concentrations (Fig. 4B). These results show that the absence of RarA partially counteracted the effect of the absence of RecU or RecX, and it reduced the maximal rate of RecA accumulation in Δ*recU* Δ*rarA* (10,000 ± 1,200 RecA /CFU) or Δ*recX* Δ*rarA* (8,400 ± 900 RecA /CFU) cells (Fig. 4A-B), suggesting that the absence of RarA counteracts the inactivation of *recU* or *recX*. This is consistent with the observation that RarA focus formation and its dymanic interaction with RecO and RecF differs from those with RecX; foci formation observed in the latter was decreased while in the other two mutant strains it was enhanced compared to *wt* cells, and additionally, it occurred earlier with regard to damage induction. This may be related to the formation of RecA-ssDNA nucleoprotein filaments, which is facilitated by RecF (16).

RarA acts as a positive contributor to RecA filament formation

To test whether RarA works as a positive modulator of RecA, its expression levels were measured upon exposing *recF*15 Δ*rarA* cells to increasing MMC concentrations (Fig. 4B). The RecA basal level of *recF15* Δ*rarA* cells was slightly lower than in the *rec*$^+$ cells (~4,100 ± 900 RecA monomers/CFU) (Fig. 4B). In the double mutant background increasing concentration of MMC failed to stimulate RecA expression (~3,900 RecA/CFU) above the RecA basal levels (Fig. 4B), suggesting that RecF and RarA might work as a alternative positive modulators. In the absence of both RarA and RecF modulators, RecA can nucleate onto SsbA-coated ssDNA by the action of RecO, but these short filaments are likely destabilized by RecX and/or RecU.

485 The estimation of the RecA basal level in the Δ*recO* Δ*rarA* strain generated uncertainties (~3200
486 ± 1900 RecA/estimated cell) due to the low viability of the Δ*recO* Δ*rarA* strain (see Fig. 1B). The
487 strain was not further analyzed.

488 **Conclusions**

489 Genetic analyses reveal that RarA acts in the context of arrested replication forks in conjunction
490 with a network of proteins that affect the activity of the RecA recombinase. Our work indicate that
491 RarA prevents uncontrolled DNA end resection and processing of stalled replication forks, with
492 subsequent fork reversion by the action of branch migration translocases (Fig. 2A-B and 3A-B).

493 Most importantly, we show that RarA positively regulates RecA filament extension, and
494 apparently counteracts the role of the negative RecA modulators. The *rarA* gene is epistatic to *recO*
495 or *recF* in response to DNA damage. There is a genetic interaction between *rarA* and *recX* and *recU*
496 because inactivation of *recU* or *recX* partially suppresses the defect of Δ*rarA* gene in response to
497 H_2O_2-induced DNA damage, but *rarA* is not epistatic to *recX* in response to MMS-induced DNA
498 damage (Fig 2B and D and 3B and D). These data are consistent with single molecule tracking
499 suggesting that one of the RarA functions is related to RecA and its accessory proteins (16). It has
500 been proposed that dynamic interactions of RarA with RecO and RecF differ from those with RecX
501 and RecU (16). When DNA is damaged, the RecA threads persist for a longer time in the Δ*recX*
502 cells (23), but there is a reduced number of RecA threads persistent in the Δ*rarA* cells (Fig. 5C). We
503 propose that RarA contributes to RecA filament extension in concert with the positive RecF
504 modulator, and both might counteract the role of the negative modulators RecX and RecU that
505 promote RecA filament disassembly, in order to protect stalled forks and prevent their degradation.
506 Our data are consitent with the observation that downregulation of FBH1, which is responsible for
507 the removal of RAD51 from chromatin, can compensate for loss of WRNIP1 activity, reinforcing

508 the hypothesis of a possible function of WRNIP1 in stabilizing RAD51 upon a direct protein-protein
509 interaction (13). Like eukaryotic WRNIP1 whose absence leads to extensive degradation of nascent
510 DNA strands (13), inactivation of *rarA* renders cells very sensitive to to H_2O_2-induced lesion, but
511 deletion of DNA end resection pathways partially suppresses the DNA repair defect (Fig. 2A and
512 3A). Our data thus show that there are strong parallels between eu- and prokaryotic RarA-type
513 proteins, and increase knowledge on the function of bacterial RarA at a molecular level. It will be
514 interesting to analyse if RarA directly interacts with RecA, or *via* a RecO-SsbA-RarA interaction
515 (see Introduction).
516

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Figures

Figure 1. Growth defects of the Δ*rarA* Δ*recO* and Δ*rarA* Δ*recA* strains. (A and B) Cells were grown in NB to reach exponential phase (OD_{560}=0.4) serially diluted, plated on NB agar, incubated ON and counted as CFU. (C) Cells were grown in NB to reach exponential phase (OD_{560}=0.4). The cells were stained with SYTO 9 (green bar) and PI (red bar) to count the number of live and dead cells respectively. Percentage of SYTO 9- and PI-stained cells are indicated. 100% corresponds to the sum of green and red cells. The results are the average of at least three independent experiments and standard errors of the mean are indicated.

Figure 2. Acute viability assays of Δ*rarA* double mutant strains upon exposure to H_2O_2. Lack of RarA in cells impaired in end resection (A), in processing of recombination intermediates (*B*), in RecA accessory proteins (C-D) or in Δ*recA* context (D). Cells were grown to reach exponential phase (OD_{560}=0.4), exposed to different concentrations of H_2O_2 for 15 min prior to serial dilutions. Cells were counted as CFU after ON growth, and results are plotted dividing these CFUs by the CFU obtained in untreated cells. The results are the average of at least three independent experiments and standard errors of the mean are indicated.

Figure 3. Acute viability assays of Δ*rarA* double mutant strains upon exposed to MMS. Lack of RarA in cells impaired in end resection (A), processing of recombination intermediates (*B*), in RecA accessory proteins (C-D) or lack RecA (D). Cells were grown to reach exponential phase (OD_{560}=0.4), exposed to different concentrations of MMS for 15 min prior to serial dilutions. Cells were counted as CFU after ON growth, and results are plotted dividing these CFUs by the CFU

745 obtained in untreated cells. The results are the average of at least three independent experiments and
746 standard errors of the mean are indicated
747
748 Figure 4. RecA protein accumulation upon SOS induction in different genetic backgrounds.
749 Exponential grown *wt* (*rec*$^+$), Δ*recX*, Δ*recU*, *recF*15, Δ*recO* and Δ*rarA* cells (A) or *wt*, Δ*recX* Δ*rarA*,
750 Δ*recU* Δ*rarA* and *recF*15 Δ*rarA* cells (B) were exposed to the indicated concentrations of MMC for
751 30 min. Then cells were collected, lysed and equivalent protein amounts subjected to 10% SDS-
752 PAGE, followed by immunoblot transfer. The number of RecA molecules/CFU are derived from a
753 standard curve of known RecA concentrations and are the average of at least three independent
754 experiments and standard errors of the mean are indicated.
755
756 Figure. 5. Time course of RecA assembly into discrete spot and extended filamentous structures
757 called "threads" after 0.5 mM H_2O_2 addition. Subcellular localization of RecA-mV after 10 min
758 intervals after H_2O_2 treatment in *wt* cells. Scale bars 5 μm.
759
760 Figure. 6. Epifluorescence microscopy showing that RecA assembly into threads is dependent on
761 RarA. (A) Subcellular localization of RecA-mV 40 min after treatment with 0.5 mM H_2O_2, in *wt*
762 (*rec*$^+$) and in Δ*rarA* mutant cells. Scale bars 5 μm. (B) Demographs of *B. subtillis* cells,
763 demonstrating the localization of RecA-mV to the middle regions. Cells were aligned and ordered
764 according to size. The fluorescence profiles represent the mean fluorescence values along the medial
765 axis after background subtraction and normalization such that the maximum fluorescence of each
766 cell is equal. C) Quantitative analysis of RecA thread formation in *wt* or *rarA* mutant cells.
767

768 **Tables**

769 Table 1. *Bacillus subtilis* strains used

Strains	Relevant genotype[a]	Source	Strains	Relevant genotype[a]	Source
BG214	*rec*$^+$	Lab. strain	BG1067	+ Δ*rarA*	(16)
BG190	+ Δ*recA*	(71)	BG1555	+ Δ*recA* Δ*rarA*	(16)
BG439	+ Δ*recO*	(56)	BG1433	+ Δ*recO* Δ*rarA*	(16)
BG129	+ *recF15*	(22)	BG1055	+ *recF15* Δ*rarA*	(16)
BG1455	+ Δ*recD2*	(32)	BG1421	+ Δ*recD2* Δ*rarA*	(16)
BG1065	+ Δ*recX*	(23)	BG1371	+ Δ*recX* Δ*rarA*	(16)
BG1337	+ Δ*addAB*	(40)	BG1107	+ Δ*addAB* Δ*rarA*	(16)
BG675	+ Δ*recJ*	(40)	BG1059	+ Δ*recJ* Δ*rarA*	(16)
BG705	+ Δ*recQ*	(40)	BG1575	+ Δ*recQ* Δ*rarA*	(16)
BG425	+ Δ*recS*	(40)	BG1563	+ Δ*recS* Δ*rarA*	(16)
BG855	+ Δ*recU*	(72)	BG1083	+ Δ*recU* Δ*rarA*	(16)
BG1131	+ Δ*recG*	(21)	BG1103	+ Δ*recG* Δ*rarA*	(16)
BG703	+ Δ*ruvAB*	(33)	BG1351	+ Δ*ruvAB* Δ*rarA*	(16)
BG1245	+ ΔradA	(31)	BG1373	+ Δ*radA* Δ*rarA*	(16)
PG5142	+ *recA-yfp*[b]	This work	PG5143	+ *recA-yfp* Δ*rarA*	This work

770 [a]All strains are derivatives of *B. subtilis* BG214 (*trpCE metA*5 *amyE1 ytsJ*1 *rsbV*37 *xre*1 *xkd*A1

771 *att*$^{SP\beta}$ *att*^{ICEBs1}). [b]RecA-mVenus is a variant of the monomeric RecA-Yfp protein.

772

773 Table 2. LD_{99} to H_2O_2 and MMS of different *Bacillus subtilis* mutant strains

Relevant genotype	LD_{99} to H_2O_2[a] in mM	LD_{99} to MMS[a] in mM	Relevant genotype	LD_{99} to H_2O_2[a] in mM	LD_{99} to MMS[a] in mM
rec$^+$	>6.0	41.2	Δ*rarA*	0.38	>50
Δ*addAB*	0.46	0.8	Δ*addAB* Δ*rarA*	4.5	44.0
Δ*recJ*	4.3	2.2	Δ*recJ* Δ*rarA*	0.47	4.6
Δ*recQ*	2.4	2.4	Δ*recQ* Δ*rarA*	1.9	4.7
Δ*recS*	4.4	2.3	Δ*recS*,Δ*rarA*	2.0	4.8
Δ*recU*	0.45	1.7	Δ*recU* Δ*rarA*	0.47	21.3
Δ*recG*	0.44	2.2	Δ*recG* Δ*rarA*	0.53	4.8
Δ*ruvAB*	0.64	4.0	Δ*ruvAB* Δ*rarA*	1.0	5.0
Δ*radA*	2.0	17.1	Δ*radA* Δ*rarA*	4.7	36.8
Δ*recO*	0.37	0.6	Δ*recO* Δ*rarA*	0.37	0.9
recF15	0.37	0.7	*recF15* Δ*rarA*	0.37	0.8
Δ*recD2*	1.9	36.6	Δ*recD2*, Δ*rarA*	0.52	43.0
Δ*recX*	0.8	10.6	Δ*recX* Δ*rarA*	0.40	7.6

774 [a]The acute lethal dose to H_2O_2 or MMS that reduced cells survival by 99% (LD_{99}) upon 15 min exposure.

775

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

H.R., S.A., P.L.G. and J.C.A designed the experiments. H.R., E.S., R.H-T., P.P.C., S.A., P.L.G. and J.C.A. planned experiments and interpreted data; H.R., E.S., R.H-T., P.P.C. and S.A. performed the experiments; H.R., E.S., R.H-T., S.A., P.L.G. and J.C.A. drafted the manuscript; and S.A., P.L.G. and J.C.A. wrote the manuscript.

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